AN INVESTIGATION OF FACTOR S, A NEUROMUSCULAR EXCITATORY SUBSTANCE FROM INSECTS AND CRUSTACEA[1,2]

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The neuromuscular function in vertebrates and in a number of invertebrates depends upon a cholinergic system. Such, however, does not appear to be true of arthropods. Repeated attempts to demonstrate cholinesterase in insect muscle have failed (Wigglesworth, 1958 and Colhoun, 1958), and such pharmacological agents as acetylcholine and paraxon and atropine have had no effect on neuromuscular transmission (Harlow, 1958; Katz, 1936; Usherwood, 1963). However, the ultrastructure of the nerve-muscle junction in several insects does suggest a chemical type of transmission (Smith and Treherne, 1963).

Several recent reports suggest that L-glutamic acid serves as an excitatory transmitter at the myoneural junction in both Crustacea and insects (Takeuchi and Takeuchi, 1964; Ozeki *et al.*, 1966; Kerkut *et al.*, 1965; and Usherwood, 1966). However, the possibility of a second substance in myoneural function is suggested by the work of Van der Kloot (1960). He found a substance in the crayfish, *Cambarus clarkii* (Girard), which stimulated the contraction of the closer muscle of the claw. This substance, called Factor S, was detected in perfusates from stimulated claws but not in perfusates from unstimulated claws. Van der Kloot extracted Factor S by the aluminum hydroxide precipitation procedure of Von Euler (1948), and he noted that this substance had several chemical properties in common with an unidentified catechol-4 isolated by Ostlund in 1954 from a number of invertebrate species.

We used the Von Euler extraction procedure on several species of arthropods in an attempt to confirm the findings of Van der Kloot and Ostlund. We have obtained a substance that has a number of properties in common with both Factor S and catechol-4.

Materials and Methods

1. Extraction procedure

All equipment and solutions used in the initial deproteinization were brought to 4° C. Whole animals or specific tissues were weighed and ground in a blender with 3 volumes of 8% trichloroacetic acid for five minutes. The homogenates were

[1] Mention of proprietary products does not necessarily imply endorsement by the United States Department of Agriculture.

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allowed to stand for ½ hour so the amines could be extracted before centrifugation at 4500 rpm for 10 minutes. The precipitated protein was discarded, and the supernatant was passed through a coarse grade, sintered glass funnel under a positive nitrogen pressure to remove non-sedimenting cuticular fragments. At this point, the supernatant was extracted with ether (1 : 1 ratio) to remove excess trichloroacetic acid and interfering lipids. A flocculate of aluminum hydroxide was then formed in the supernatant by first adding 1 ml. of 20% aluminum sulfate for every 100 ml. of solution and then adding 0.5 N sodium hydroxide (with constant stirring) until a pH of 7.5 was reached. The aluminum hydroxide was removed from the solution by centrifugation at 4500 rpm for 10 minutes. The precipitate was then dissolved in a minimum amount of N sulfuric acid. Sufficient 0.5 N sodium hydroxide was added to bring the solution to a pH of 2.8. Extraneous salts were removed from the solution by adding 4 volumes of ethanol. This mixture was allowed to stand for 15 hours at 4° C. to complete the precipitation process. The salts were removed by centrifugation, and the resulting supernatant was taken to dryness *in vacuo* at 40° C. Further desalting of the residue was usually necessary and was accomplished by the addition of 100 ml. of acetone. The mixture was then allowed to stand on ice for 45 minutes to complete the process, the salts were discarded, and the supernatant was again taken up in 10 ml. of 0.5 *M* sodium phosphate buffer (pH 6.5) and placed on a cation exchange resin.

2. Chromatographic purification of Factor S

An Amberlite IRC 50 cation exchange resin was prepared in the sodium form described by Bergström and Hansson (1950). This ion exchanger separated the catecholamines from organic acids, sugars, and natural pigments occurring in crude extracts. The column containing the crude extract was eluted with 75 ml. of 0.2% sodium chloride and then by 75 ml. of 1 N HCl. The N HCl fraction from the column was taken to dryness, and the residue was dissolved in 0.2 ml. of ethanol. The eluate was then spotted on Whatman No. 1 filter paper with a micropipet, and the solvent was removed by a stream of nitrogen. The spotted papers were chromatographed in a phenol-0.1 N HCl system (1 : 1) in a descending system for 18 hours at 24° C. ± 1°.

Initially, the position of Factor S was determined by bioassay; later, spray reagents such as potassium ferricyanide and naphtho-quinone-4-sulfonate were used. Generally, the active area from the phenol system was eluted and rechromatographed in a butanol-acetic acid-water system (4 : 1 : 5) before assay.

Physiological Preparations

1. Isolated ventral nerve cord

Adult male American cockroaches, *Periplaneta americana* (L.), were decapitated, opened from the dorsal surface, and pinned ventral side down on a piece of cork. The thoracic portion of the nerve cord was removed and placed on recording electrodes. The preparation was perfused with the saline solution described by Twarog and Roeder (1957). The electrodes were connected to the push-pull input of a Grass P-5 preamplifier. A Hewlett-Packard oscilloscope was employed to monitor endogenous activity. The most frequently observed response in un-

treated preparation consisted of a steady but arrhythmic background of action potentials that ranged in amplitude between 30 and 100 μv. These potentials ranged from 50 to 1000 cps in frequency (Fig. 2a). This level of activity remained constant for several hours. For comparison purposes, a preparation of the abdominal nerve cord of the crayfish was used. Figure 2f shows normal activity on such an isolated nerve cord. With such preparations, Van Harreveld's solution was used to perfuse the isolated cord while it was in contact with the electrodes.

2. Motor nerve preparation

An *in situ* preparation of the cockroach motor nerves was used to bioassay the substance. Adult males were decapitated, slit along the dorsal midline from the last abdominal segment through the prothoracic segment, and pinned ventral side down on a cork platform. The region surrounding the metathoracic ganglion was exposed, and all nerves leading to the ganglion except the abdominal and forward thoracic connectives were severed. Fine platinum electrodes were placed underneath the severed ends of nerves 5 or 6. The activity of such preparations was observed for 10 to 15 minutes until activity was considered steady. Samples of Factor S were then placed on the ganglion with a micropipet.

3. Nerve-muscle preparation

Male cockroaches were decapitated and pinned dorsal side down through the prothoracic and 6th abdominal segments to a paraffin-filled petri dish. The sternal sclerites of the metathoracic segment were removed to expose the ganglion, and the fifth was exposed more extensively by removing the cuticular membrane running between the coxa and the sternite. The anterior of the coxa was then rotated 90° to bring it into a ventrally upright position. The coxa was held in this position with insect pins and plasticene. A Grass mechanical electrical transducer was then oriented so the extensor muscles of the coxa, by moving the trochanter, would push against the extended hook of the transducer. This arrangement (Fig. 1) provided an essentially isometric situation. The transducer was connected to a Grass P-6 DC preamplifier, and the output was fed to an oscillograph or chart recorder. All nerves leaving the ganglion were severed, and two platinum electrodes were placed beneath the fifth nerve for indirect stimulation of the extensor muscles of the coxa. The four major extensor muscles in the coxa innervated by the fifth nerve are, according to the nomenclature of Carbonell (1947), muscles 178, 179D, 177E, and 179 (all these muscle units are attached to the large extensor tendon). The distal anterior surface of the coxa was removed to permit the profusion of test solutions into the muscle elements.

Results

Biological Properties of Factor S

1. Isolated ventral nerve cord

Regions on the paper chromatograms that contained the active principle were eluted with 95% ethanol, taken to dryness, and dissolved in 1 ml. of physiological saline solution. The concentration of Factor S in the solution was then estimated

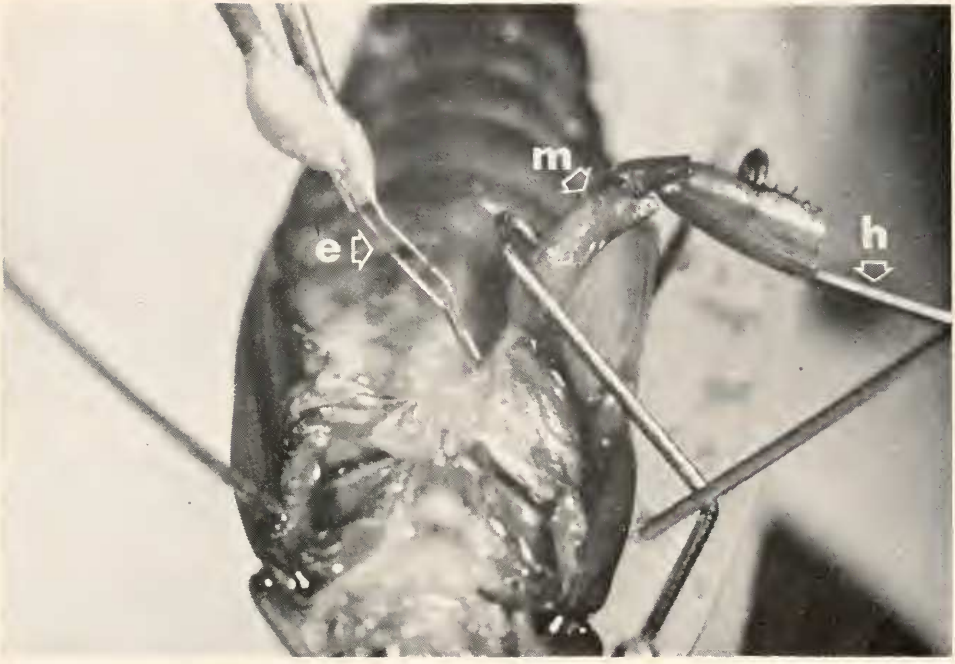

FIGURE 1. The cockroach nerve-muscle preparation. E = stimulating electrodes under the severed fifth nerve; H = hook from the transducer; M = exposed muscle elements.

by taking 100-μl. aliquots to dryness and weighing them on a Cahn electrobalance until a constant weight was obtained. This weight was corrected for salt content and trace contaminants from paper. In general, whole animal extracts were used as a source of Factor S. However, a number of extracts from specific tissues were evaluated. The activity of such extracts showed no difference from Factor S obtained from whole animals. In each experiment, the observation of endogenous activity on the isolated nerve cord for 10–15 minutes before applying Factor S constituted a control (Fig. 2a). Figure 2a through 2d shows a typical experiment with Factor S obtained from a whole animal extract of the American cockroach. Within 1 minute after the application of a solution containing 18 μg. of residue, the thoracic nerve cord of the cockroach showed a marked rise in arrhythmic activity (Fig. 2b). Also a two- to three-fold increase in pulse amplitude was usually observed. This excitatory response normally continued from 4 to 7 minutes and was succeeded by a rapid decline in spontaneous activity to a level considerably below normal (Fig. 2c). The cord finally became electrically silent within 5 to 10 minutes (Fig. 2d). Ordinarily, the nerve cord returned to normal levels of activity after 30 to 45 minutes in fresh saline solution. The induction of neural blockade was more rapid when the solution contained larger amounts of active residue (35 μg. or more). Also, the initial rise in arrhythmic activity was not apparent, and the blockade was more persistent. Two to three hours in fresh saline solution were required for recovery, and by that time, the nerve cord usually exhibited abnormal activity.

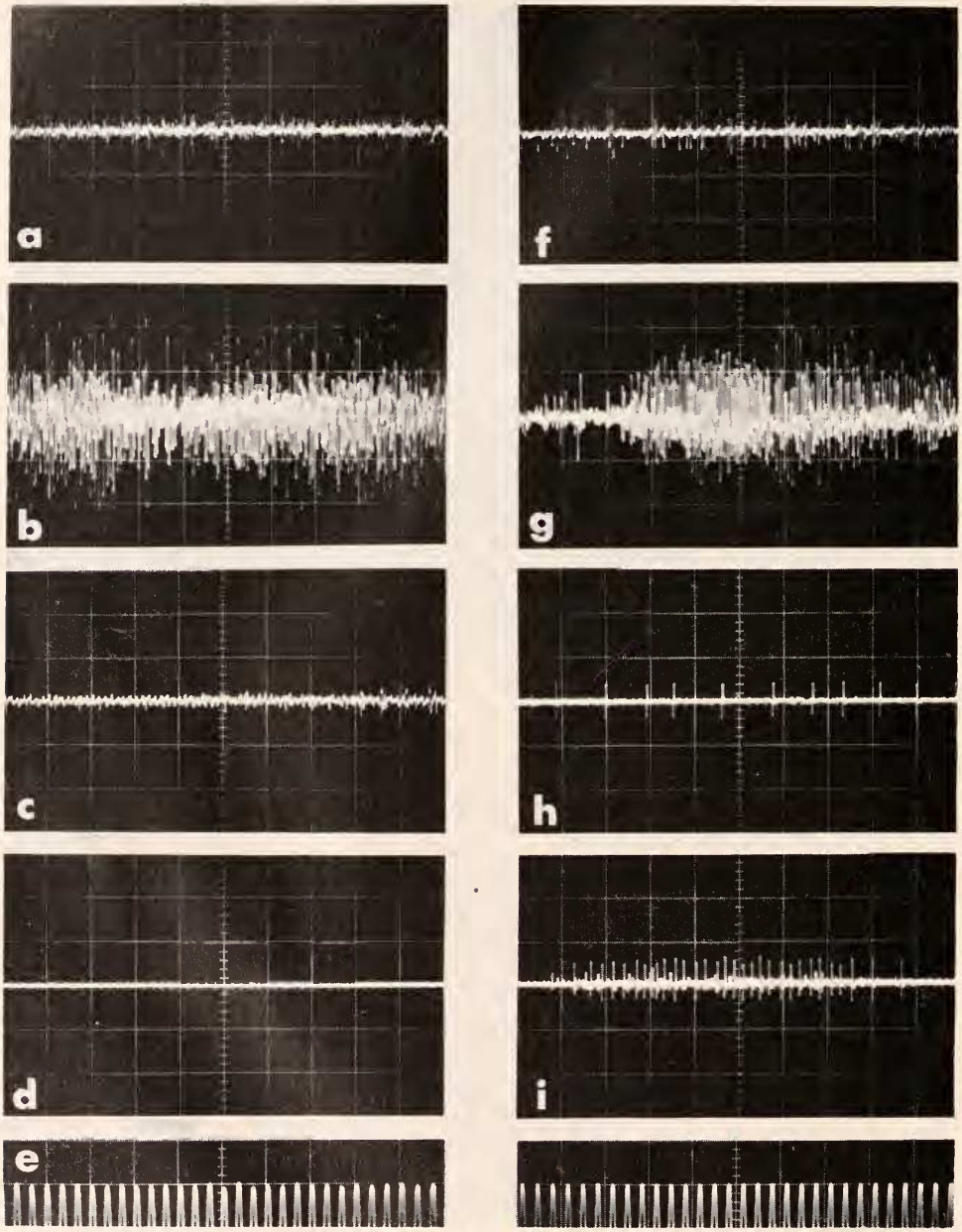

FIGURE 2. The response of the isolated nerve cord and motor nerve preparations to extracts containing Factor S: (a) normal activity on the isolated thoracic nerve cord of the cockroach; (b) same as (a) 30 seconds after the application of 18-μg. residue of an active sample; (c) same as (a) but 4 minutes after application of the 18-μg. residue; (d) blockade of the central nervous system 10 minutes after initial application of the 18-μg. residue sample; (e) time signals, 60 cps; (f) normal spontaneous activity of the isolated abdominal nerve cord of the crayfish in

The threshold for neural excitation with Factor S appeared to be low. The type of response recorded in Figure 2b was observed in a number of experiments with saline solution containing less than 8 μg. of residue. Active extracts obtained from the whole crayfish also excited the isolated abdominal nerve cord from the same animal (Fig. 2g). Saline solution containing 18 μg. residue induced an immediate rise in arrhythmic activity, then a depression, and finally blockade within 10 to 15 minutes.

Factor S not only increased the level of arrhythmic activity in nerve preparations, but also induced large bursts of high frequency pulses. Such bursts (Fig. 2g) were intermittent and had seemingly unpatterned recurrence. Frequently in isolated nerve cords, a large complex burst of activity occurred the instant the active sample came into contact with the preparation. Also, treated nerve cords often showed a marked facilitation after any kind of mechanical stimulation, such as that created by a drop of fresh saline solution.

The biological action of Factor S does not appear to be species-specific among arthropods. Active extracts from the American cockroach were just as effective on the isolated abdominal nerve cord of the crayfish as Factor S obtained from the crayfish itself, and vice versa.

2. Motor-nerve preparation

Normal activity from the motor fibers of the fifth nerve at the metathoracic ganglion is shown in Figure 2h. Figure 2i shows the response of these same nerve fibers two minutes after 15 μg. of residue was applied to the metathoracic ganglion. Impulse trains such as those shown in Figure 2i were repetitive, recurring every 2 to 3 seconds. With several preparations, muscular contractions in the mesothoracic segment occurred, and these contractions were coincident with the trains of impulses shown in Figure 2i.

3. Nerve-muscle preparation

Since Van der Kloot stated that Factor S had an excitatory effect on crustacean muscle, a number of experiments were made with a cockroach nerve-muscle preparation to determine what effects our active extracts might have. Figure 3 shows the potentiating effect of an active extract from the cockroach on the extensor muscles of the trochanter after a 30-second perfusion; in this instance, the concentration of Factor S is estimated at less than 20 μg. Active extracts from the house fly, *Musca domestica* (L.), produced a similar response.

4. Occurrence and tissue distribution of Factor S

Factor S was found in whole animal extracts of the crayfish *C. clarkii,* and the following insect species: the cockroaches *P. americana, Leucophaea maderae* (F.), and *Blaberus giganteus* (L.); the grasshopper, *Schistocerca vaga;* and the house fly, *M. domestica.* The active principle was present in the head, ventral nerve

physiological saline; (g) same as (f) 30 seconds after exposure to a sample of Factor S (18 μg. of residue); (h) normal activity of the motor neurons in the severed 5th nerve from the metathoracic ganglion; (i) same as (h) 2 minutes after the application of 20 μg. residue of Factor S preparation to the ganglion.

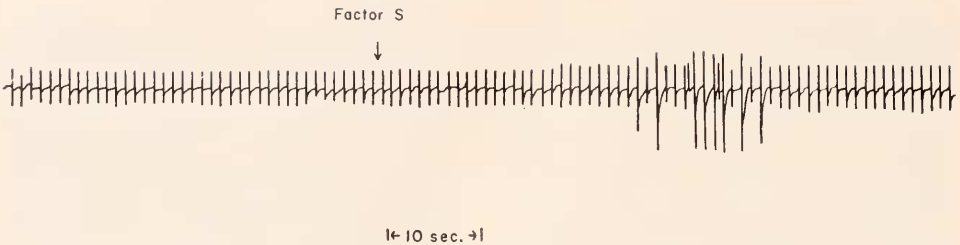

FIGURE 3. Recording of the mechanical response of the extensor muscles of the trochanter in the cockroach. Normal response to the application of a pulse strength of 6 volts every second is shown at the left. Potentiation in this response is seen at the right after a 30-second perfusion with Factor S.

cord, and the legs of the American cockroach as determined by bioassay and chemical spot tests.

The biological activity of 1-g. extracts of the ventral nerve cord were nearly equivalent to that found in 300-g. extracts of the whole animal, suggesting a high specific activity for Factor S in nervous tissue.

Chemical Properties of Factor S

1. *Extraction and purification by chromatography*

Extracts were generally prepared from 300 to 400 g. of whole insects or crayfish. Although the extraction procedure of Von Euler was normally used, several alternate methods of extraction were tried. Extraction with perchloric acid (Van der Kloot, 1960) was successful, and the active principle was also readily removed from tissues with 5% HCl in ethanol.

Initially paper chromatography was the simplest means of isolating Factor S from crude extracts. The active principle had an R_f of 0.55 to 0.65 on chromatograms developed in a butanol-acetic acid-water system and an R_f of 0.02 to 0.09 in the phenol-HCl system. Unfortunately, these chromatograms from extracts of insects and crayfish were generally streaked with a reddish-brown pigment and invariably contained a large number of fluorescent compounds. These interfering substances were removed by passing crude extracts through a 1×10 cm. column of IRC 50 before paper chromatography, as described previously. The initial 75 ml. of 0.2% sodium chloride contained the reddish-brown pigments and naturally fluorescent material; Factor S and such catecholamines as dopamine were eluted in the N HCl fraction. The catecholamines were readily separated from Factor S by paper chromatography in the phenol. In this system, Factor S never exceeded an R_f value of 0.1, and norepinephrine, the slowest migrating catecholamine, always had an R_f greater than 0.2 (Fig. 4).

When ethanol eluates from the phenol system were rechromatographed in butanol-acetic acid-water, the R_f of Factor S was found to be 0.15 to 0.2 (Fig. 4) in contrast to the earlier values obtained when crude extracts were simply spotted on paper. The higher R_f values were, in all probability, the consequence of co-chromatography with an unknown ortho-diphenol since elutions from this R_f region often showed a major absorption band at 280, which is characteristic of

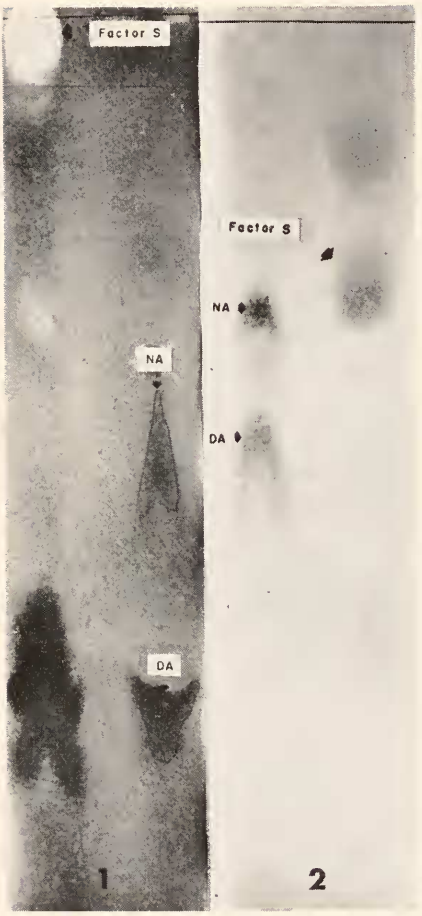

FIGURE 4. Paper chromatograms showing the position of Factor S; origin line is at the top of the photograph. (1): Factor S on chromatogram developed in phenol, 0.1 N HCl (1:1) and treated with potassium ferricyanide and Prussian Blue as reagents. (2): Factor S on chromatogram developed in butanol-acetic acid-water (4:1:5) and treated with 1,2-naphthoquinone-4-sulfonate. NA = norepinephrine; DA = dopamine.

many ortho-diphenols (Kamlet, 1957). This 3-phase sequence of chromatography has been used routinely to prepare samples of Factor S for chemical and biological tests. Although the purity is by no means assured, it is an improvement over previously reported isolations.

When Factor S was chromatographed in the butanol-HCl system, it again had a low R_f value (between 0.1 and 0.2) just below norepinephrine. The chromatographic behavior of Factor S in the three systems suggests a rather polar compound.

2. *Chemical reaction*

Chromatograms containing Factor S were sprayed with a number of chemical reagents. The results of these tests for specific functional groups are summarized

TABLE I

Functional group analysis of Factor S from the American cockroach as determined by organic spot tests

Reagent	Reaction	Functional group
Potassium ferricyanide in phosphate buffer (pH 7.7)	pink (purple UV)	a catechol or catecholamine
Ammoniacal silver nitrate	gray	a phenol, polyol, or indol
Diazotized *p*-nitroaniline	reddish-pink	a phenol or aromatic amine
2-Butanone + ammonia	negative	not a 1-substituted nicotinamide
Formaldehyde + KOH	negative	not a monoamine with a hydroxyl group in the meta position
Ethylenediamine + ammonia	negative	not an ortho-diphenol
Ninhydrin	negative	not a primary or secondary aliphatic amine
Sakaguchi test	negative	not a guanidine
Na 1,2-naphthoquinone-4-sulfonate	orange-brown	a reactive NH_2 or CH_2 group
p-Dimethylaminobenzaldehyde + conc. HCl	yellow	a primary aromatic or aliphatic amine
Diazotized sulfanilic acid	negative	not an imidazole, phenal, or aromatic amine

in Table I; the method of preparing and applying the reagents was taken either from Block *et al.* (1958) or Feigl (1960). Chromatograms sprayed with potassium ferricyanide gave a pink color in visible light and under ultraviolet light a deep blue to purple fluorescence in the region of biological activity. This reaction, for some reason, was much more consistent and intense in the phenol system than in the other developing solvents. If the chromatograms treated with potassium ferricyanide were sprayed with Prussian Blue, a bright bleached spot appeared against a blue background in the region containing Factor S (Fig. 4). The chemistry and significance of this reaction are not clear; however catecholamines generally reacted when this material was used as a reagent to give a deep blue spot against a light blue background (Fig. 4).

The potassium ferricyanide spray has perhaps had its widest use as an oxidizing agent in the detection of such catecholamines as epinephrine, norepinephrine, and dopamine (James, 1948). These amines form quite distinct and intense fluorescent products after treatment with this reagent. However, potassium ferricyanide is not specific for catechols since it can react with glucosamine and galactosamine to yield a pink color and purple fluorescence.

When areas containing Factor S were reacted with ammoniacal silver nitrate, they gave a gray color that is characteristic of phenols, polyols, and indols. The negative tests with ethylenediamine and ammonia and alkaline formaldehyde strongly suggested that Factor S is not an ortho-diphenol. Occasionally, active samples in saline solution showed a strong absorption band at 270 mμ. However, the addition of the borate ion to such a sample did not cause a bathochromic shift in the absorption. Di-ortho-substituted catecholamines generally give such a shift in the presence of the borate ion (Jurd, 1957). Norepinephrine hydrochloride, for example, when placed in insect saline solution, undergoes a 7-mμ bathochromic shift in the presence of this ion. The positive results obtained with naphthoquinone-

4-sulfonate and *p*-dimethylamino-benzaldehyde strongly suggested the presence of a primary amine.

3. *Solubility and stability*

Factor S is readily soluble in water and ethanol but not in chloroform or diethyl ether. The active principle appears to be stable in both hydrochloric and acetic acid, and it is unaffected by 10-minute exposures to an alkaline environment (pH 9). A stream of air bubbled through saline solutions containing Factor S for 50 minutes showed no detectable loss in biological activity, which suggests that it is not readily susceptible to oxidation. However, active samples in saline solution taken to dryness with an infrared lamp (75° C. for 40 minutes) lost their activity when they were resuspended. The elevated temperature does not seem to be the causative agent since active samples showed no change in the intensity of their biological response after being placed in boiling water for 10 minutes. When a 1-ml. solution of Factor S was incubated for 45 minutes at 37° C. with 35 μg. of chymotrypsin, no loss in activity was observed. Generally, preparations of Factor S retained their activity from 7 to 10 days in physiological saline solution held at 4° C. Occasionally, such samples were active for 3 to 4 weeks after storage.

Although the majority of analytical tests made to determine the chemical structure of Factor S were performed on the American cockroach, sufficient chromatographic and chemical data were obtained on the other species to permit the assumption that the material extracted from them was identical.

Discussion

Factor S, as described by Van der Kloot (1960), had an R_f of 0.6 and 0.05 in the butanol and phenol systems, respectively; it reacted with potassium ferricyanide to give a pink color and with naphthoquinone-4-sulfonate to give a yellow color. The substance we extracted from insects and crayfish had these same chemical and chromatographic properties. Van der Kloot further stated that Factor S gave a yellow color on exposure to cyanogen bromide vapor, followed by a spray of 1% *p*-aminoacetophenone in ethanol. He also observed a white fluorescence after treatment with cyanide fumes or the vapors of ammoniacal 2-butanone. On the basis of these latter tests, Van der Kloot proposed a 1-substituted nicotinamide structure for Factor S. However, the fluorescence that he observed is difficult to interpret since he made no mention of observing the natural fluorescence that is evident in extracts of both crayfish and insects. Also, ammonia alone caused either fluorescence or an increase in fluorescence in many materials that appeared in the extracts. Thus, the combination of ammonia and 2-butanone was probably not a specific test. In the cyanogen bromide p-aminoacetophenone test, we observed a number of materials in extracts which reacted with *p*-aminoacetophenone to give a yellow color, even though they had not had previous exposure to cyanogen bromide, again an indication of a lack of specificity.

In an attempt to further substantiate the 1-substituted nicotinamide hypothesis, Van der Kloot injected crayfish with 0.5 μc. of C^{14}-labeled nicotinic acid. Twenty-four hours after treatment, these animals were extracted and chromatographed on paper. When he scanned these chromatograms, he found a peak of radioactivity coincident with the R_f for Factor S. Efforts by Armson and Horridge (1964) to

repeat this phase of the work were unsuccessful; they found that metabolites formed from radioactive nicotinic acid had no effect on neuromuscular transmission in the crayfish or the crab. Their evidence and the negative results we obtained with ammoniacal 2-butanone on purified samples strongly suggests that Factor S is not a 1-substituted nicotinamide.

Van der Kloot mentioned that the only substance that he could find in the literature comparable to Factor S was catechol-4 isolated by Ostlund (1954). This substance, like Factor S, was extracted by its adsorption properties on aluminum hydroxide. Ostlund found catechol-4 in several insects, the protozoan, *Noctiluca miliaris* (Suriray), the coelenterate, *Metridium dianthus* (L.), and the mollusk, *Mytilus edulis* (L.), and treatment of these chromatographed extracts with potassium ferricyanide produced a pale rose-colored spot at a low R_f in the butanol-HCl (1:1) system. However, the R_f value of this spot was not consistent with norepinephrine, epinephrine, dopa, dopamine, tyrosine, or 5-hydroxytryptamine, and the specific nature of the extraction procedure ruled out acetylcholine and histamine. When catechol-4 was chromatographed in phenol saturated with 0.1 N HCl, it had an R_f of 0.02 and 0.03 and again gave a rose color with potassium ferricyanide. Under UV light, it showed a blue color with a faint bluish-white fluorescence. The only biological property of catechol-4 observed by Ostlund was its distinct hypotensive effect upon the fowl rectal caecum. He found considerable amounts of catechol-4 in the hagfish heart, which is completely insensitive to varying doses of epinephrine, norepinephrine, dopamine, tyramine, and acetylcholine.

Our chemical results with Factor S agree with Ostlund's in almost every detail. The substance had the same R_f in butanol-HCl and phenol-HCl; it also gave a positive reaction with potassium ferricyanide and a negative one with ninhydrin.

There are three reasons for dismissing an ortho-diphenol structure for Factor S: (1) The active principle is unable to form a fluorescent condensation product with either ethylenediamine or formaldehyde. (2) No bathochromic shift in the UV spectra of active samples was observed in the presence of the borate ion. (3) The stability of Factor S in an alkaline environment is in marked contrast to the properties of most catecholamines.

Active samples were incubated with chymotrypsin since the UV spectra of the samples suggested the possibility of a peptide with aromatic amino acid residues. However, no loss in biological activity was observed after such treatment, implying that Factor S is not a peptide. This assumption is further supported by the negative tests with ninhydrin. Factor S is chemically, chromatographically, and biologically distinct from L-glutamic acid, γ-amino-butyric acid, and 5,6-dihydroxytryptamine. At the moment, the only positive information available on the chemical nature of Factor S is suggested by its solubility and the spot tests with dimethylaminobenzaldehyde and naphthoquinone-4-sulfonate. These findings indicate the presence of an amine function. The possible presence of an aromatic ring in the active principle is implied by the positive tests with diazotized *p*-nitroaniline and ammoniacal silver nitrate as well as the phenolic type absorption occasionally observed in UV.

One might speculate that Factor S potentiates neuromuscular transmission by simply increasing the number of quanta released per stimulus as 5-hydroxytrypta-

mine does in Crustacea (Grundfest *et al.,* 1959). Prospects of such a circumstance existing, however, appear unlikely for several reasons: (1) We have observed a high specific activity of Factor S in insect neural tissue. (2) Factor S has been found in perfusates from indirectly stimulated muscles of the crayfish (Van der Kloot, 1960).

Conclusions

The substance we extracted from insects and crayfish parallels closely the biological and chemical properties of Factor S and catechol-4. Each of these substances has been designated a biogenic amine for one reason or another, and it is our contention that they are all closely related chemically, if not identical. Clearly, Factor S is implicated in the neuromuscular function of arthropods. The substance elicits an excitatory response from motor neurons in the cockroach, and it potentiates the contractions of the extensor muscles of the trochanter at low concentrations. Such features suggest that Factor S might function as a better motor transmitter in arthropods than L-glutamate; however, a more intensive physiological investigation at the cellular level is necessary to evaluate the full significance of these findings.

Since the chemical purity of Factor S is uncertain, the results of functional group and spectral analyses must be accepted with a degree of caution. Trace contamination can readily hinder organic reactions by blocking key groups in the active molecule. Results may also be obscured by the sensitivity of organic reactions. Spot tests are known to vary within wide limits, sometimes as much as 100-fold.

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Summary

1. A neuroactive substance has been found in extracts of the crayfish, *Cambarus clarkii* (Girard) and the following insect species: the cockroaches, *P. americana* (L.), *Leucophaea maderae* (F.), and *Blaberus giganteus* (L.), the grasshopper, *Schistocerca vaga,* and the house fly, *M. domestica* (L.). This substance in low concentrations excites motor neuron activity in the American cockroach. It also potentiates the mechanical response of the indirectly stimulated extensor muscles of the trochanter in the same insect. There is also a high specific activity of the excitatory agent in the central nervous system of the cockroach.

2. Data from extraction, chromatogaphy, and chemical analyses suggest a biogenic amine. This amine appears distinct from all commonly known neuropharmacologically active agents.

3. The chemical and biological similarities between this substance and van der Kloot's Factor S and Ostlund's catechol-4 are discussed.

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